RESEARCH PLAN PROPOSAL

TERATOLOGICAL EVALUTION OF DICOFOL (ORGANOCHLORINE) AND DELTAMETHRIN (SYNTHETIC PYRETHROID) IN CHICK EMBRYO

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Topic

Teratological evaluation of dicofol (organochlorine) and deltamethrin (synthetic pyrethroid) in chick embryo.

Introduction

Researchers are becoming more inclined to anticipated problems posed by the chemical pesticides not only in this country but worldwide (Imelda *et al.* 1994). Pesticides are major contaminants of our environment and many persist in environment including in various feed and foodstuffs. Approximately 85,000 tons of pesticides per year are used in India. Currently 400 members of three groups of pesticides (organophosphates, organochlorines and synthetic pyrethroids) are being used in India. About 40 % of these are organochlorines (which include endosulfan, dicofol, aldrin, dieldrin, lindane, isodrin, heptachlor). Many pesticides continue to flow into the market despite government restrictions and farmers prefer many of these chemical pesticides because they are cost effective, are easily available, and display a wide spectrum of bioactivity (Garg *et al.* 2004).

The term "pesticide" refers to any substances used to control something which has been designated as a "pest"-itself another term, covering a diverse array of organism. Insects are probably the most common type of pest encountered and as a group; they are arguably the most destructive globally (Worthing, 1991). The pesticides usage has contributed for increasing agriculture production and suppressed the vectors of health diseases, but sometimes due to wrong identification of pests and diseases, the farmers will not apply suitable chemical which in turn will create pollution problems.

The organochlorine (chlorinated hydrocarbon) insecticides are diverse group of agents belonging to three distinct chemical classes including the dichloro-diphenylethane, the chlorinated cyclodiene, and the chlorinated benzene and cyclohexane related structures (Ecobichon, 2001). These insecticides were used extensively in all aspects of agriculture and forestry, in the building and structural protection to control a wide variety of insect pests. The properties like low volatility, chemical stability, lipid solubility, slow rate of biotransformation and degradation made these chemicals such effective insecticides also bought about their demise because of persistence in environment, bioconcentration, and biomagnifications (Ecobichon, 2001) within various food chains and the acquisition of biologically active body burdens in many wildlife species that, if not lethal certainly interfered with reproductive success of the species(Garg *et al.*2004). They operate by disrupting the sodium/potassium balance of nerve fiber, forcing the nerve to transmit

continuously. These insecticides are potent inhibitors of Na^+, K^+-ATPase and more importantly, the enzyme Ca^{2+}, Mg^{2+}-ATPase that is essential for transport (uptake and release) of calcium across the membranes. The inhibition of Ca^{2+}, Mg^{2+}-ATPase, located in the terminal ends of neurons in synaptic membranes, results in an accumulation of intracellular free calcium ions with promotion of calcium induced release of neurotransmitters from storage vesicles and subsequent depolarization of adjacent neurons and the propagation of stimuli throughout the Central Nervous System(CNS) (Tomlin, 1997). Their toxic effects in animals are principally due to hyper excitation in the nervous system and death is frequently ascribed to respiratory failure after the disruption of nervous system.

Dicofol (2, 2, 2-trichloro-1, 1-bis (4-chlorophenyl) ethanol), an organochlorine pesticide is a miticide that is very effective against red spider mite. Dicofol is usually synthesized from technical Dichloro Diphenyl Trichloroethane (DDT). During the synthesis, DDT is first chlorinated to an intermediate, Cl-DDT, and then hydrolyzed to dicofol. After the synthesis reaction, DDT and Cl-DDT may remain in the dicofol product as impurities. Dicofol is structurally similar to DDT. This has caused criticism by many environmentalists; however, the World Health Organization classifies dicofol as a Level III, "slightly hazardous" pesticide. It differs from DDT by the replacement of the hydrogen (H) on C-1 by a hydroxyl (OH) functional group. Dicofol first appeared in the scientific literature in 1956, and was introduced into the market by the US-based multinational company Rohm & Haas in 1957. Other current manufacturers include Hindustan (India), Lainco (Spain), and Makhteshim-Agan (Israel) (Tomlin, 1997). The US Environmental Protection Agency (EPA) temporarily canceled the use of dicofol because relatively high levels of DDT contamination were ending up in the final product. Modern processes can produce technical grade dicofol that contains less than 0.1% DDT. Dicofol is a nerve poison. In mammals it causes hyper stimulation of nerve transmission along nerve axons . This effect is thought to be related to the inhibition of certain enzymes in the central nervous system. Symptoms of ingestion and/or respiratory exposure include nausea, dizziness, weakness and vomiting; dermal exposure may cause skin irritation or a rash; and eye contact may cause conjunctivitis. Poisoning may affect the liver, kidneys or the central nervous system. Very severe cases may result in convulsions, coma, or death from respiratory failure. It can be stored in fatty tissue. Intense activity or starvation may mobilize the chemical, resulting in the reappearance of toxic symptoms long after actual exposure. The primary effects after long term exposure to dicofol include increase in liver weight and enzyme induction. It is known to be harmful to aquatic animals and can cause eggshell thinning in various species of birds.

Synthetic pyrethroids are synthesized derivatives of naturally occurring pyrethrins which are taken from pyrethrum, the oleoresin extract of dried *Chrysanthemum* flowers. The insecticide properties of pyrethrins are obtained from ketoalcoholic esters of Chrysanthemic and pyrethroic acids (Mueller-Beilschmidt, 1990).These are strongly lipophilic and rapidly penetrate many insects and paralyze their nervous system (Reigart and Roberts, 1999). Pyrethrins affect the nervous system of insects by causing multiple action potentials in nerve cell by delaying the closing of an ion channel (Bradberry *et al.* 2005).

Deltamethrin was synthesized in 1974, and since then, it has been applied for a range of commercial crops and recreational uses, and by extension controls a variety of pests. It is used primarily on cotton, coffee, maize, cereals, fruits, and stored products. Deltamethrin is also applied in animal health and public health capacities. Chemically, it is the [1R, *cis*; alpha S]-isomer of 8 stereo isomeric esters of the dibromo analogue of chrysanthemic acid, 2, 2-dimethyl-3-(2, 2-dibromovinyl) cyclopropanecarboxylic acid (Br_2CA) with alpha-cyano-3-phenoxybenzyl alcohol. Deltamethrin is considered the most powerful and therefore the most toxic of the Pyrethroids, up to three orders of magnitude or more than some (Extoxnet, 1996). In the studies done on workers in agricultural settings, Deltamethrin can produce a variety of acute health conditions such as writhing syndromes, convulsions, and salivation, ataxia, dermatitis, diarrhea, tremors, and vomiting. Allergic reactions to the compound through skin exposure are also common among agricultural workers. The chemical also carries several ecological risks, particularly by causing algal blooms that can in turn harm fish and other aquatic life through clogging gills and decreasing the water's level of oxygen. It also reduces bee populations and their associated pollination service. Deltamethrin is highly stable in air and sunlight because of its strong adsorption on particles once exposed to either, it does not degrade, even after two years' time at 40° Celsius and pose risks to mammals and the ecosystem as a whole, (Extoxnet, 1996) Both the World Health Organization and the United States Environmental Protection Agency list Deltamethrin as moderately hazardous, with the WHO labeling the compound as a Type II Acute Hazard. Under laboratory conditions, Deltamethrin has been found to be highly toxic to a range of aquatic organisms such as amphibians, crustaceans, mollusks and various forms of plankton (EPA, 1998).

Though beneficial to the agriculture sector and in vector borne disease control, the Organochlorines and synthetic Pyrethroids cause toxicological effects such as illness and death in animals. These toxicological effects arise from various circumstances, either direct or indirect animal contact with pesticides.

Experiments with pesticides are conducted to assess the risk in humans. The avian egg and developing embryo have been used to test for developmental toxicity and other effects for a longer period of time because ; the morphological changes that occur during chick embryogenesis are very similar to those of other vertebrates, the large size of the embryo and *in vivo* development makes it accessible to surgical and biochemical manipulation and easy to observe embryos at different stages and follow them through developmental changes, each embryo develops in self –contained environment, free from the maternal variables of nutrition and placental dysfunction, the ability to manipulate the precise number of eggs to use as well as the exact dose to administer to each egg provides increased control of experimental procedure and easy to obtain chick egg and embryo year round because of short 21- day gestation (Jelinek 1982; Kotwani 1998).

Review of Literature

A number of environmental concerns have been raised by use of pesticides which are generally applied for increasing crop yield and reducing post harvest losses. One disaster at a pesticide manufacturing plant was in Bhopal, India. The plant accidentally released 40 tons of an intermediate chemical gas, methyl isocyanate, used to produce some pesticides. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than target species including non target species, air, water and soil. The World Health Organization and the UN Environment Programme estimate that each year, 3 million workers in agriculture in the developing world experience severe poisoning from pesticides, about 18,000 of whom die (Miller, 2004). According to one study, as many as 25 million workers in developing countries may suffer from acute health problems, such as abdominal pain, dizziness, headaches, nausea, vomiting, as well as skin and eye problems (Ecobichon, 2001). Additionally, many studies have indicated that pesticide exposure is associated with long-term health problems such as respiratory problems, memory disorders, dermatologic conditions, cancer, depression neurological deficits, miscarriages, and birth defects.

In birds, pesticides results in physiological effects at several levels, including direct effects on breeding adults as well as developmental effects on embryos. The effects on embryos include mortality or reduced hatchability, failure of chicks to thrive, and teratological

effects producing skeletal abnormalities and impaired differentiation of the reproductive and nervous systems through mechanisms of hormonal mimicking of estrogens (Fry, 1995).

Among the pesticides, organochlorine compounds are well known for their long persistence in environment (Vettorazzi, 1975) and living organisms (Gowdar and Sethunathan, 1976).Some types of organochlorides have significant toxicity to plants or animals, including humans. Dioxins, is produced when organic matter is burned in the presence of chlorine, and some insecticides such as DDT. Pesticides, as they are known today, originated with the discovery of the insecticidal properties of 1, 1-bis (p-chlorophenyl)-2, 2, 2-trichloroethane (DDT) in 1949. The impact of this compound was astonishing for the wildlife. Hickley (1969) stated the peregrine falcon was disappearing owing to reproductive failures due to exposure of DDT. These reproductive failures were characterized by delayed breeding, failure to lay eggs, thinning and breaking of shells or failure to produce more eggs after earlier clutches were lost, and a high mortality of embryos and fledgling(Swartz, 1981).

Analytical screening of wildlife tissue samples for organochlorine chemicals rarely includes dicofol, and this may explain why, compared with other organochlorine, the relative hazard of dicofol to wildlife populations is poorly known (Clark and Flickinger, 1995). Dicofol is 'highly' to 'very highly' toxic to a range of aquatic organisms, including fish, invertebrates and estuarine/marine organisms (EPA, 1998). In birds, dietary concentrations of dicofol between 1 and 10 mg/g (wet weight) fed to captive adult females caused eggshell thinning, reduced hatching success, or reduced fertility in American kestrels *(Falco spar-veruis)* and eastern screech-owls *(Otus asio)* (Clark and Flickinger, 1995).

According to Collin (1987), Dareste (1877) referred to study on chick embryo, but Ancel's work (1950) appears to have been the first report of malformations induced in the chick embryo with chemical agents. Karnofsky (1952; 1955), one of the pioneers of the field, made a systemic study of toxic and teratological effects on chick development of various substances, particularly metals. He selected the fourth day of incubation for injecting test compounds in the yolk. This day was chosen so that developmental defects could be induced that were compatible with embryo survival on day 18.

The effect of phenoxy herbicides 2, 4-D, 2, 4, 5-T, MCPA, mechlorprop and dichlorpop on hatchability of hen eggs and the viability of the chicks was investigated both by injecting and immersing the eggs in 1 and 5 % solution of the herbicides. These herbicides were

found to have rather similar embryotoxic effects. Injection of herbicides decreased the percentage of hatch and in some cases the viability of the chicks. Immersion in herbicides had moderate effect on hatchability of the eggs and the viability of the chicks. The embryotoxic effect of 2, 4-dichlorodibenzo-p-dioxin was found to be least 100 times that of the herbicides. In the injection experiments a considerable number of malformations were observed in dead embryos at high dose levels (Hansen and Mikkelsen, 1974).

The effects of carbaryl dissolved in two vehicles (acetone and sesame oil) on chick embryos following 5 and 12 days of exposure were examined. Swartz (1981) reported that groups of embryos exposed to carbaryl showed a significant increase in mortality. There were no indications of external abnormalities in any of 5 day surviving embryo; however, a greater embryotoxicity were seen in embryos exposed to carbaryl in sesame oil for 12 days. Subcutaneous edema was a common defect seen in embryos receiving the higher dosages of carbaryl in acetone.

Hoffman (1981) reported that environmental contaminants-xenobiotics can cross the shell and its membranes and are subsequently taken up by avian embryo on direct exposure. The presence of pollutants in the developing avian egg has been shown to result in decreased hatchability and increased neonatal death.

Teratogenic effects of two organophosphate insecticides, diazinon and dicrotophos, were investigated by Misawa *et al.* (1982) in regard to skeletal development, particularly of the extremities and vertebrae. Diazinon and dicrotophos inhibited growth of the following skeletal elements: femur, tibia, metatarsi and digits of the leg. The greatest reduction of the skeletal length was observed in tibia and metatarsi, and was characterized by angulations toward the dorsal side. In the cervical region of embryos treated with the insecticides, unique deformities such as an “undulating”; notochord and fused cervical rings were seen at an early stage of development.

The toxicity of 3,3’,4,4’-tetrachloroazobenzene (TCAB) and 3, 3’, 4, 4’-tetrachloroazoxybenzene (TCAOB) in the chick embryo was examined by Schrankel *et al.,* (1982).The time of injection had a major effect on embryo mortality as eggs injected with TCAB or TCAOB on the fourth day of incubation had higher incidence of mortality than eggs injected on day 11-13. Both TCAB and TCAOB showed the toxic effects. Rump edema was the major abnormality observed in embryos treated with either TCAB or TCAOB. Other malformations included altered feather pattern and lack of down feather, hemorrhage, external viscera, reduced body size, failure to withdraw the yolk sac, beak malformation, dilation of blood vessels, and monomicrothalmia.

White Leghorn chicken eggs were treated with the organophosphate insecticide dicrotophos and early defects thus induced were characterized by Garrison and Wyttebbach (1985). Treated embryos displayed general developmental retardation as well as unilateral retardation of cranial sense organs. Eggs injected at 48 or 72 hr displayed notochordal folding, usually restricted to cervical region and deformities in cervical region. Other defects seen were branching of the neural canal in lumber region, bifurcation of the neural epiphysis, deformation of the lens vesicle, and the distension of the major blood vessels. The incidence and severity of epiphyseal, lens, and vascular defects were greatest among embryos treated at 24 hr, whereas notochordal and both types of neural defects were greatest among 48 hr. All these observed changes diminished with increasing age such that by 96 hr the only defect noted was a weak notochordal folding in one embryo.

Teratological effects of fungicide maneb (ethylenebisdithiocarbamate) were observed by Maci and Arias (1987) in chick embryos. Unincubated eggs were immersed in different concentration of fungicide aqueous solution for 30 sec. Treated eggs were incubated for 19 days. In their investigation they reported that single treatment with maneb was teratogenic at all concentration tested, producing mainly unilateral lower limb deformities such as bent tibia, tibiotarsus and phalanges.

Elawar (1990) investigated the effects of carbamates when injected in ovo to chick embryos, at two time periods (days 5 and 15) during incubation. Carbaryl dosed at 45 mg kg-1 egg weight was extremely toxic to the embryos on day 5 of incubation. Hatchability was reduced to 0% as compared to 80% when carbaryl was injected on day 15 of incubation. Aldicarb at 1.5 mg kg-1 egg weight had no major effect on hatchability when injected either on day 5 or day 15 of incubation (hatchability = 90 and 100%, respectively). Brain acetylcholinesterase (AChE) and liver cholinesterase (ChE) were inhibited significantly during incubation in embryos dosed on day 15 with both carbaryl and aldicarb. Liver carboxylesterase was inhibited significantly during incubation with only the carbaryl treatment. All esterase enzyme activities returned to normal after hatching. The locomotion of chicks was affected in both treatment groups until 47 days after hatching.

Kumar and Devi (1992) investigated the effect of methyl parathion (MP) on developing chick embryos. The embryos were exposed at 4^{th}, 6^{th} and 9^{th} day of incubation to various doses (5, 10 or 50µg) of MP. On 20^{th} day of incubation embryos were examined for any teratogenecity. Observed embryos showed retarded growth which included reduced body weight and reduced body length and length of leg bones. Other teratogenic signs observed were short neck, muscular hypoplasia of legs, abdominal hernias and hemorrhages spots in brain and upper body.

Varnagy (1998) studied the degradation of pesticides parathion, methylparathion, carbendazim, 2, 4-D-amine Na, phosmethylane in hen eggs. Eggs were treated by immersion technique on day 9 or 12 of hatching period. The residues of pesticides were measured in samples on days 13, 14 and 16 of incubation of chicken. Analytical chemistry data showed a varying degradation rate of the compounds. The residues directly affect the embryos, disturbing their normal development and causing pathological and morphological changes.

Pourmirza (2000) investigated the toxic effects of malathion and endosulfan on chicken eggs. The LD_{50} values of these insecticides proved malathion to be more toxic than endosulfan. He reported that combined action of malathion and endosulfan causes increased mortality and reduced embryonic body weight in comparison with action of each individual compound alone.

The embryotoxicity of the insecticide primicid in chick embryo were studied by Khalil and El- Sayed (2000).The eggs were immersed, for 30 seconds, in the concentration of 0.25, 0.50, 1.0, 2.0 and 4.0% aqueous solutions, before incubation. On day 16 of incubation, the eggs were opened and examined for embryotoxic effects. No significant differences were obtained in weight between different treatment groups. Furthermore, primicid resulted in dose-related increase in mortality rate and induced external malformations such as hydrocephaly, deformed beak, shorter hind limbs parts and haemorrhagic spots in the hindbrain and abdomen.

The effect of dimecron, an organophosphorus insecticide, on developmental alterations and histopathological damage was determined by Sahu and Ghatak (2002) in the developing chick embryo. The insecticide was administered at two different doses (25 µg and 35 µg) into the egg yolk at day 0 of incubation. Significant abnormalities in relation to organogenesis (eye defects, crossed beak, abnormally exposed brain and internal organs and limb defects) and overall retardation in growth were noted in the insecticide-treated embryos at day 4, 8 and 14 of incubation. Histopathological study of the treated whole embryo showed abnormal features in the formation of different vital organs. The liver was severely affected by the pesticide at both the doses. Observed liver cells were non nucleated, vacuolated and ruptured, causing obliteration of the sinusoids. Deshaped and pycnotic nuclei were also prominent.

Varga *et al.* (2002) made a study to investigate whether broadly applied insecticides fenitrothion was able to penetrate the avian eggshell following exposure to Sumithion 50 EC (50 % fenitrothion) by immersion. Residues were quantified in yolk and embryonic

samples, and two widely used routes of exposure (injection and immersion) were compared in terms of residues. In their study they demonstrated that fenitrothion was able to cross the eggshell after external exposure and contaminate the developing embryo with penetration rate that increased after the mobilization of Ca from the eggshell. The fenitrothion residues measured in the samples indicated that the embryos were contaminated by the chemical for a longer time after external exposure than administration of insecticide by injection.

Anwar (2003) investigated toxic effect of cypermethrin on the development of chick. The study included the investigation of teratological and biochemical changes in the developing embryos. Among biochemical constituents, activities of the few enzymes and some biochemical content of whole embryo were investigated. He reported that activity of amylase increased whereas as the activity of ALP (alkaline phosphatase) decreased. However, the activities of ACP (Acid phosphatase), ALT (Glutamate pyruvate Transaminase), AST (Glutamate oxaloacetate transaminase) and LDH (Lactate dehydrogenase) remained unaltered. Of the biochemical components, glycogen, free amino acids, total lipids, cholesterol, DNA and RNA contents were seriously affected. Total protein, soluble protein, uric acid and urea contents also remained unaffected with cypermethrin treatment. Glycogen content, cholesterol content, total lipid content and DNA showed a significant increase at low dose level and significant decrease in high dose level. Free amino acid content was decreased. Teratological changes observed included reduction in crown rump length, the size of the brain and size of eyeballs, incomplete development of eyes, beak and wing buds, micromelia, exocardiogenesis. In some animals, eyes and beak were totally absent.

The toxicity of single sublethal doses of various concentrations (25, 50, 100 and 200 ppm) of permethrin insecticide on the liver of 16-day old chick embryo was investigated by Anwar *et al*. (2004). Permethrin was injected in to the eggs on day '0' of incubation. Results showed that the activities of alkaline phosphatase (ALP), acid phosphatase (ACP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were significantly decreased, whereas the activity of amylase remained unchanged. From among biochemical components glucose, glycogen, total protein, soluble protein, urea and RNA contents were significantly decreased, while total lipids and free amino acid content were significantly elevated. However, cholesterol, uric acid and DNA contents remained unaltered with permethrin treatment. Permethrin-induced histopathological changes in liver showed increased sinusoidal spaces in hepatic

parenchyma, cytoplasmic vacuolations in hepatocytes, hepatocytic nuclear condensation, fatty degeneration, hydropic degeneration and necrosis of hepatocytes.

Alhifi *et al.* (2004) studied the teratogenic effects of dimethoate on chick embryo. He reported that dimethoate causes deleterious effects and shows significant abnormal developmental effects on the heart (mispositioned or malformed), brain (compacted brain, microcephaly or macro cephaly), neural tube and somites during the organogenesis and it inhibits the activity of AChE (Acetylcholinesterase) enzyme.

Teratological tests were carried out by Keseru *et al.* (2004) on avian embryos. A 30% dimethoate containing insecticide formulation (BI 58 EC) and a 20% benfluralin containing herbicide formulation (Flubalex) and a 960 g/l s-metolachlor containing herbicide formulation (Dual Gold 960 EC) were studied in chicken embryos after single administration by immersion and injection technique. Treatment was done on day 0 of incubation. Test materials were injected into the air chamber in a volume of 0.1 ml/egg, or eggs were treated by the immersion technique for 30 min at 37 degrees C. Evaluation was done on day 19 of incubation. Injection treatment: the administration of s-metolachlor and benfluralin did not result a significant decrease in the average body weight of embryos. At the same time the body weight of embryos significantly decreased because of single administration of dimethoate. The embryo mortality increased markedly after the administration of test materials (s-metolachlor, benfluralin, and dimethoate). Immersion treatment: the administration of s-metolachlor, benfluralin and dimethoate did not result a significant decrease in the average body weight of embryos. The rate of embryo mortality was low after the administration of these pesticides.

A 50% dichlorvos containing insecticide formulation (Unifosz 50 EC) and a 50% atrazine containing herbicide formulation (Hungazin PK 50 WP) were studied in chicken embryos after administration as single compounds by Szabó *et al*. (2004). Applied concentrations of dichlorvos were 0.1% (corresponding to the plant protection practice), 0.05%, 0.02%, 0.01%. Applied concentrations of atrazine were 0.66% (corresponding to the plant protection practice), 0.33%, 0.132%, 0.066%. The test materials were injected directly into the air-chamber of eggs on day 0 of the hatching period and evaluation was carried out on day 19 of incubation. After the single administrations of dichlorvos containing insecticide formulation and atrazine containing herbicide formulation on day 0 of incubation, the average body weight of chicken embryos significantly did not decrease as compared to the control. After the individual administrations of pesticides the incidence of developmental anomalies was sporadic. The embryonic mortality markedly increased at the highest concentrations of pesticides. The rate of embryo mortality was 61% (dichlorvos insecticide

containing formulation) and 52% (atrazine containing herbicide formulation). They concluded that the 50% dichlorvos containing insecide formulation (Unifosz 50 EC) and the 50% atrazine containing herbicide formulation (Hungazin PK 50 WP) were toxic to the developing chicken embryos at the highest concentration.

Rachid *et al.* (2008) investigated the effects of the flufenoxuron, which is an Insect growth Regulator (IGR) on embryonic development of hen's eggs. The pesticide is tested *in ovo* by injection in the egg air cell of three concentrations (1, 10 and 20 µg/egg) in strictly controlled conditions, to show the impact on some biochemical parameters. Results showed that the 20µg concentration induces a great level of embryonic mortality in the developing chick embryos. The significant decreasing of blood serum proteins, cholesterol and Alanine aminotransferase (GPT) and increasing of triglycerides was also observed at this dose level of flufenoxuron.

Ethanol exposure is also toxic to chick embryo. The study was conducted by Kamran *et al.* (2009) to test the effect of ethanol vapour exposure on the survival of chick embryos. From their investigation they concluded that ethanol vapour exposure decreased embryo survival with increasing embryonic age and increased duration of exposure.

Petrovova *et al.* (2009) investigated toxicity of bendiocarb (2, 3-isopropyledene-dioxyphenyl methylcarbamate) to the organs of chicken embryo. The toxic action of bendiocarb was observed on liver and central nervous system (CNS). The observations showed no macroscopic or microscopic changes in the liver and CNS with either dose or day of incubation when the bendiocarb was administered. The liver and CNS were also investigated for caspase activity in relation to application of bendiocarb and no differences in the number of cells with caspase immunopositivity were observed in comparison with the control.

Developmental toxicity of two different classes of commercial formulations of insecticides was studied by Uggini *et al.* (2010) in fertilized Rhode Island Red eggs. The first one was a combination of chlorpyrifos and cypermethrin and the second one was spinosad, a fermentation product of soil bacterium, *Actinomycetes*. In this study, the combination pesticide and spinosad of different concentrations were administered as a single dose *in ovo* in volumes of 50 µL per each egg on day ''0'' of incubation. They reported that the combination insecticide induced explicit alterations in the embryonic growth and development and resulted in malformations particularly to the axial and appendicular skeletal structures (such as crooked legs, twisted phalanges, beak deformities, microphthalmia, anophthalmia, wry neck, craniorachischisis, deformities in formation of

sternum and ribcage, vertebral deformities, micromelia, missing phalanges, and umbilical hernia etc.), whereas the changes were trivial in case of the spinosad exposure.

Petrovova *et al.* (2010) observed embryotoxicity of cholinesterase inhibitor bendiocarb in the chick embryo. The pesticide dissolved in 10% acetone in water for injection was applied in a volume of 200 µl over the embryo through membrana papyracea at embryonic days (ED) 2, 3, 4, 5 and 10. The toxicity of bendiocarb was rather low, and lethal dose (LD (50)) decreased with advancing development from 0.97 mg per egg at ED 2 to 28.6 mg on ED 5. The malformations in surviving embryos were observed rarely (<2%) and occurred in both control and experimental groups. In the treatment at ED 5 and 10 there was a statistically significant reduction in body weight, but the maximum difference from controls was below 14%. In treated chick embryos on ED 3 and 4, small but not significant increase in number of dead cells using supravital staining was observed.

Mobarak and Al-Asmari (2011) demonstrated the effects of the organochlorine pesticide endosulfan (35% EC) on the developing chick embryos. After 24 h of eggs incubation, a single dose of 7 or 14 or 21 mg endosulfan/egg was administered through the egg air space at once. The eggs were opened on embryonic days 6 and 12 and the embryos were evaluated for viability, wet body weights and various morphological, morphometric and skeletal changes. Comparing the three doses with control and with each others, the high dose treatment resulted in statistically significant more embryonic deaths, while the mid-dose caused statistically more malformed embryos. On both embryonic days, the treated embryos exhibited dose-related growth retardation, as reflected by significant reductions of embryonic wet body weight, anterior-posterior head and crown-rump lengths as well as generalized edema and hematomas formations. Also, on embryonic day 12 significant reductions of beak length, eye diameters and measurements of wing and hind-limb parts were recorded. Abnormal survivors showed high percentages of limb deformities (as limb paralysis, clinodactyly, flexion and shortness of limbs or digits), microphthalmia, microtia and omphalocele. The skeleton of treated embryos showed anomalies and incomplete chondrification and/or ossification of some skull parts (interorbital septum, frontals, parietals, palatines and external auditory apertures), cervicals, scapulae, ribs, sacrals and caudals.

The chronic effect of pesticides mixture (dimethoate 30% and methidathion 40%) on the AChE of developmental embryo of *Gallus gallus domesticus* was investigated by Alhifi (2011). The lethal concentration of pesticides mixture for 50% killing (LD50) values was found to be 40 ppm. Embryos were injected with pesticides mixture of 1/5th LD50 and 1/10th LD50 (8 and 4 ppm) on each alternative day starting from incubation day 7 for 2

weeks. On day 21 after 12 hours of the last dose an amount of 200 µl blood was collected from the blood vessels surrounding the embryonic membranes and the heamolyzate was used for the assessment of the AChE activity calorimetrically. Result of this study indicated that 1/10th of the LD50 had only marginal effect on the AChE activity (40.6%). Whereas 1/5th of the LD50 of pesticides mixture caused significant inhibition of AChE activity (69%) which could not be reversible.

Motivation/Justification and Relevance

Most pesticides are potent killer. Though not instantaneous in effects, sub lethal doses may produce long term results that may even be worse than death.

- Importance of study is that it helps to know the various lethal/toxic effects of these pesticides on laboratory animals at different dose level.
- Research involving laboratory animals is necessary to ensure and enhance human and animal health and protection of the environment.
- In the absence of human data, research with experimental animals is the most reliable means of detecting important toxic properties (teratogenicity and embryotoxicity) of these chemical substances and for estimating risks to human and environmental health.
- Teratological tests carried out on laboratory animals facilitate the development of environment-friendly chemical plant protection techniques.
- Findings from this work will provide addition information and knowledge in field of developmental toxicology.

Objectives

The present study aims for:-

- The analysis of teratogenicity in chick embryo caused by xenobiotics; Dicofol and Deltamethrin.

Plan of Work and Methodology

Toxicants

- **Dicofol:** Organochlorine pesticide with trade name COLONEL- S (18.5% EC).
- **Deltamethrin:** Synthetic pyrethroid pesticide with trade name DECIS® (2.8% E.C).

Experimental Subjects

Fertilized eggs of BV 300 breed (*Gallus domesticus)* will be collected from a poultry farm from Ajmer and will be kept in an incubator with capabilities of maintaining and monitoring temperature, humidity and turning the eggs periodically. The temperature in the incubator will be maintained at 38 $\pm 0.5^0$C and relative humidity will be kept between 70-80%.

Doses

Different doses of toxicants will be prepared according to recommended dose based on field application. There will be three doses for each toxicant-

1. Low dose: Concentration of toxicant that will be half of the recommended dose.
2. Medium dose: Concentration of toxicant that will be equal to the recommended dose.
3. High dose: Concentration of toxicant that will be double of the recommended dose.

Mode of administration of dose

Experimental eggs will be administrated different doses of each toxicant or vehicle by immersion technique (dipping for 1 hour at 37°C temperature).

Groups of Experimental Subjects

Eggs will be divided into three groups based on nature of solution in which they will be immersed-

1. Group I (Untreated Group): A predefined number of non manipulated eggs will be served as Control Group I to study background toxicity.
2. Group II (Vehicle treated): Alike number of eggs will be immersed in vehicle (distilled water) in which different doses of toxicants will be prepared. This group will be served as Control Group II.
3. Group III (Toxicant treated): A predefined number of eggs will be immersed in different doses (low, medium and high) of toxicants.

Experimental design

There will be three sets of experiments

- The dose will be given on "0" day of incubation period and autopsies will be done on 4^{th} day and 7^{th} day of incubation for teratological and biochemical studies. Histopathological study (liver), biochemical study (brain and liver) and Alizarin red

stain preparation for the study of skeletal deformities will be done on embryos taken out on 16th day of incubation.

- The dose will be given on 4th day of incubation and autopsies will be done on 7th day and 10th day of incubation for study of teratological and biochemical parameters. Histopathological study (liver), biochemical study (brain and liver) and Alizarin red stain preparation for the study of skeletal deformities will be done on embryos taken out on 16th day of incubation.
- The dose will be given on 7th day of incubation and embryos will be taken out on 16th day of incubation for histopathological study (liver), biochemical study (brain and liver) and Alizarin red stain preparation for the study of skeletal deformities.

Experimental plan

Experiment 1

Fertilized eggs prior to incubation (0day)
(30 fertilized eggs in each experiment)

↓

Will be dipped in three doses (low, medium, high) of each pesticide on the "0" day of incubation period for 1 hour.

↓

67% of embryos will be taken out on 4th day and 7th day of incubation to study teratological and biochemical parameters both.

↓

Remaining 33 % of embryo will be sacrificed on 16th day of incubation for histopathological study (liver), biochemical study (brain and liver) and Alizarin red stain preparation for studying skeletal deformities. (10 embryos for each autopsy will be taken).

Experiment 2

Fertilized eggs on 4th day of incubation
(30 fertilized eggs in each experiment)

↓

Will be dipped for 1 hour in three doses (low, medium, high) of each pesticide on 4^{th} day of incubation.

↓

67 % of embryos will be taken out on 7^{th} day and 10^{th} day of incubation to study teratological and biochemical parameters.

↓

Remaining 33 % of embryo will be sacrificed on 16^{th} day of incubation for histopathological study (liver), biochemical study (brain and liver) and Alizarin red stain preparation for studying skeletal deformities. (10 embryos for each autopsy will be taken).

Experiment 3

Fertilized eggs on 7^{th} day of incubation
(10 fertilized eggs in each experiment)

↓

Will be dipped for 1 hour in three doses (low medium, high) of each pesticide on 7^{th} day of incubation.

↓

All embryos will be sacrificed on 16^{th} day of incubation for histopathological study (liver), biochemical study (brain and liver) and

Alizarin red stain preparation for studying skeletal deformities. (10 embryos for each autopsy will be taken).

Alike number of eggs will receive vehicle only in which dose concentration will be made and will be served as controls. An untreated group of eggs will accompany control group. These control groups will be autopsied on the same day as the treated for comparing the results. Each experimental plan will be repeated in triplicate.

Parameters

Following parameter will be studied for each experiment:-

- **Teratological parameter**

  Survival rate

  Number of malformed embryo

  Incidence of morphological malformation (head, beak, eye, neck, limb, lower body and general growth retardation)

  Incidence of skeletal malformation (skull, vertebrae, ribs, sternum, upper limb and lower limb)

- **Biochemical parameters**

  Protein content- Lowry *et al.* (1951)

  Glycogen- Rex-Montgomery (1957).

  Cholesterol- Liebermann-Burchard reaction (Henry and Henry1974).

  DNA content and RNA content by the methods of Schneider (1957) using diphenylamine and orcinol reagents, respectively.

  Activities of enzymes

  - ✓ Alkaline Phosphatase and Acid phosphatase by method of Kind and King (1954).
  - ✓ Glutamate oxaloacetate transaminase and Glutamate pyruvate transaminase method of King (1965).
  - ✓ Reduced Glutathione (GSH) Moron *et al.* (1979).
  - ✓ Acetylcholinesterase (AChE) Ellman *et al.* (1961).

- Histopathological study of liver.
- Alizarin Red S preparation will be made in eggs opened on 16^{th} day of incubation for the study of skeletal deformities.

Techniques

The following techniques will be used in present proposed research:-

Alizarin red stain preparation: Skeletal deformities in 16 day old chick will be studied by technique described by McLEOD (1980).

Centrifugation: It is one of the most important separation techniques. It is based on the principle of sedimentation. The substances will be isolated by application of centrifugation field. We will use this technique for homogenate preparation of chick embryo.

Spectrophotometery: It is technique used to determine the concentration of a chemical in a solution. If solution has a color, Spectrophotometer will measure the intensity of color and relate the intensity of color to the concentration of solution. This will be used in present study for analysis of all biochemical parameters such as total protein, lipid, cholesterol, enzymes activities, DNA and RNA contents of chick embryo.

Microtomy: Technique will be used for studying histological, histopathological and histochemical details of liver in which thin paraffin sections (up to 5µ size) of liver will be cut with a microtome.

Statistics

Statistical analysis will be done on parametric values of chick embryos which will be expressed as mean± standard error (S.E). The experimental values will be compared with those of corresponding controls; the statistical significance of variance will be calculated by using **student "t" test.** Non parametric changes (survival data and number of embryos with one or more malformations) will be compared by **Mann- Whitney "U" test**. The significance level will be obtained from table of significance provided. The value of *p* as 0.05, 0.01, and 0.001 will be considered to be significant, highly significant and very highly significant respectively.

Place of work and Facilities available

Proposed research work will be undertaken in the Department of Zoology, The IIS University, Jaipur, Rajasthan. All the facilities and resources like egg incubator, spectrophotometer, all other instruments and chemicals required for this research work will be provided by the department.

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